EXHIBIT A

Immune Deviation Strategies in the Therapy of Psoriasis

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Abstract: The experience with biologicals in currently available animal models suggest that inflammatory autoimmune disease depend on IFN-γ-producing T helper (Th) cells. Deletion of T cells improves most of these autoimmune diseases but bears the risks of general immunosuppression. Alternatively, selective deviation of the inflammatory, disease-inducing Th cells into an anti-inflammatory Th cell phenotype may be a promising strategy to treat inflammatory autoimmune diseases, such as psoriasis, rheumatoid arthritis, multiple sclerosis or autoimmune diabetes. The common feature of these organ-specific autoimmune diseases is the close association with IFN-γ-producing Th1 cells, which recognize organ-specific antigens and orchestrate the cells and mediators that ultimately cause the tissue damage. Even though the autoantigens recognized in psoriasis remain enigmatic, it has been the first Th1-mediated autoimmune disease successfully treated in humans by immune deviation. The basis of such an immune intervention therapy has been established in experimental mice with model diseases of multiple sclerosis, rheumatoid arthritis or autoimmune diabetes. In all these autoimmune diseases clinical improvement was associated with the skewing of IFN-γ producing autoantigen-specific Th1 cells into an IL-4 dominated Th2 phenotype. Such Th2 cells are still reactive to the autoantigen but provide a different cytokine pattern. The most powerful cytokines capable of inducing anti-inflammatory Th2 cells are IL-4 itself or IL-11. Interestingly, another agent that has been used for decades in the therapy of psoriasis in some European countries, fumaric acid esters (FAE), seems also to induce immune deviation. This review focuses on the potential immune deviating strategies based on the use of IL-4, IL-11 or FAE in the therapy of psoriasis, the effects of these agents on the immune system, potential risks and future perspectives for therapeutic intervention by immune deviation replacing immunosuppression.

T CELLS IN INFLAMMATORY AUTOIMMUNE DISEASE

Inflammatory autoimmune diseases affect 3-5 % of the population and can manifest in multiple organ systems of the body. The most frequent inflammatory autoimmune diseases that target one specific tissue are type I diabetes, autoimmune thyroiditis, multiple sclerosis, uveoretinitis, rheumatoid arthritis or psoriasis. Various cells of the immune system are involved in the pathogenesis of these diseases and a number of experimental animal models helped fundamentally to understand the immunological processes leading to T cell-mediated or immunglobulin (Ig)-mediated autoimmune disease. One of the best established mouse models to investigate the role of lymphocytes in organ-specific autoimmune disease is experimental autoimmune encephalomyelitis (EAE), which mimics human multiple sclerosis. Based upon the studies on the role of $CD8^+$ T cells in the control of infections or tumor defense, cytotoxic $CD8^+$ T cells were suggested to be the cells mainly responsible in the development of inflammatory autoimmune diseases. Even though CD8 knock out mice ($CD8^{-/-}$) showed a milder course of disease in the acute phase of EAE, surprisingly, a higher frequency of relapses has been observed in the chronic phase of the disease [1]. In parallel to the studies on $CD8^+$ T cells, several groups focused on the role of $CD4^+$ T cells as the predominant autoreactive lymphocytic population in the pathogenesis of autoimmune diseases. Using a rat EAE model, a therapeutic approach with anti-CD4 antibodies has been shown to be effective [2]. In the following specific $CD4^+$ T cell clones have been identified, which can induce EAE after transfer into naive mice. These T cell clones are reactive to synthetic peptides of myelin-derived antigens [3]. Cytokine analysis of $CD4^+$ T cells recognizing myelin-derived peptides showed an IFN-γ-producing Th1 cell phenotype that release little or no IL-4 [4,5]. More importantly, autoreactive Th1 cells induce severe disease while Th2 cell clones or lines had non encephalitogenic potential [5,6]. Analysis of disease development and progression in other experimental diseases such as models of autoimmune diabetes, proteoglycan-induced arthritis or experimental autoimmune uveoretinitis support the concept that these diseases are mediated by autoreactive IFN-γ-producing Th1 cells [7-9].

TH1 - TH2 DIFFERENTIATION

Naive $CD4^+$ T cells can differentiate into two major subpopulations, IFN-γ-producing Th1 cells or $CD4^+$ T cells of a Th2 phenotype, which is characterized by the secretion of large amounts of IL-4 and IL-5 but only small amounts of IFN-γ. This antagonistic polarization is dependent on at least two stimuli: stimulation of Th cells through the T cell receptor (TCR)/CD3 complex and a cytokine. IL-12 is the cytokine that strongly promotes the generation of Th1 cells

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while IL-4 induces Th2 development during activation through the TCR [10-13]. Importantly, the most potent Th2 inductor, IL-4 itself, can deviate even recently activated antigen-specific Th1 cells into an anti-inflammatory Th2 phenotype [14,15]. Functionally, Th1 cells mediate delayed type hypersensitivity reactions, protect against intracellular pathogens or control tumors [16-19]. In contrast Th2 cells create an anti-inflammatory environment and are essential for effective immune reactions against extracellular parasites or for the initiation of humoral immune responses [20,21]. Compared to the early insights into the central role of Th cell subpopulations in infectious diseases, the role of Th cell polarization for the development of autoimmune diseases has long been debated. Over the past ten years it became increasingly evident that autoreactive Th1 cells are the cells inducing EAE, autoimmune diabetes or arthritis, whereas Ig-mediated autoimmune diseases seem to be associated with autoreactive Th2 cells [5,22-26].

IMMUNE DEVIATION VERSUS IMMUNOSUPPRESSION

The therapy of autoimmune diseases is still primarily based on immunosuppressive agents such as corticosteroids, cyclosporine or methotrexate. These agents are very effective in many patients with inflammatory autoimmune diseases. Because of the general immunosuppression provided by these agents they may promote the risk of infections and tumor development [27,28]. Immunosuppression was originally viewed as an antigen-specific therapy that would be based on 'silencing peptides'. Such peptides would compete with the pathogenic peptide. Such approaches with altered peptide ligands of myelin basic protein have been tested in patients with multiple sclerosis [29,30]. Until today, these specific therapies failed and current treatment of autoimmune diseases is primarily based on conventional immunosuppressive drugs [31]. Alternatively, new biologicals were designed that selectively inhibit defined T cell populations, such as anti-CD4 or anti-CD3 antibodies [32-34].

An alternative approach would be deviation of disease-inducing Th1 cells into an autoreactive anti-inflammatory Th2 phenotype. Animal experiments suggest that such an approach may be helpful to treat inflammatory autoimmune diseases. Two signals are important for the induction of Th2 cells *in vitro* as well as *in vivo*. Stimulation of the TCR by the autoantigen and IL-4, which can be provided by paracrine secretion from simultaneously activated Th2 cells or by exogenous delivery of IL-4 [35,36]. Immune deviation by continuous subcutaneous injections of IL-4 was first analyzed in the experimental model of EAE and showed therapeutical success. Subsequently, other experimental models such as autoimmune diabetes or collagen-induced arthritis have been investigated [5,23,24]. Continuous three times daily injections of IL-4 seem to be essential in order to improve typical DTH reactions, such as contact hypersensitivity reactions [16]. In all these preclinical models, improvement was closely associated with the induction of antigen-specific Th2 cells. Since IL-4 has also been shown to worsen the clinical course of these diseases under certain experimental conditions, it is important to note that the therapeutic effect of IL-4 is strongly depending on its dose, duration and time of application [37,38]. In order to induce Th2 cells, IL-4 must be given during T cell activation. When IL-4 is present prior to T cell activation, IL-4 paradoxically can induce an IL-12-producing DC phenotype which in turn primes for Th1 responses [37,39].

PSORIASIS AS A T CELL MEDIATED AUTOIMMUNE DISEASE

Psoriasis is a chronic inflammatory autoimmune disease of the skin and joints, which affects 2-4% of the Caucasian population. Several factors are needed in the immunopathogenesis of psoriasis, which finally leads to the proliferation of keratinocytes and endothelia and the clinical manifestation of psoriatic plaques. These factors include predisposing factors, such as certain human leucocyte antigens (HLA) and psoriasis susceptibility genes as well as environmental and psoriasis triggering factors, such as streptococcal infections [40,41]. These factors may interact with the immune system and promote disease activation. Beside T lymphocytes, several other immune cells, such as mast cells, macrophages, Langerhans cells and neutrophils seem to be involved in the inflammatory process leading to the clinical manifestation of psoriasis [42,43]. The exact interactions between these immune cells via surface molecules or soluble factors are still poorly understood. Together, the inflammation results in a hyperproliferation of keratinocytes and endothelia, which are the most prominent signs of a psoriasis plaque. T lymphocytes are essentially needed for the development of a psoriasis plaques. Together, these findings resulted in a concept, where psoriasis is considered to be a Th1-mediated autoimmune disease [27,44,45]. This is supported by several clinical observations.

Two drugs which are currently used in the systemic therapy of psoriasis, cyclosporine and methotrexate, have important immunosuppressive effects [31,46,47]. In patients with psoriasis who underwent allogeneic bone marrow transplantation for hematological malignancies, psoriasis plaques disappeared after transplantation of bone marrow from non-psoriatic donors. These patients tend to remain free of psoriasis even after discontinuation of the immunosuppressive medication [48]. On the other hand reports exist on the unexpected, first development of psoriasis in transplant patients, who received bone marrow from psoriatic donors [49]. Clinical studies show that antibodies against CD3 or CD4 or T-cell selective immunotoxins can clinically improve psoriasis [32-34,50]. On the other side, cytokine studies unraveled that Th1 cytokines also aggravate psoriasis. In the eighties psoriasis was thought to have an infectious origin, as IFN-γ was found locally in suction blister fluid from psoriatic lesion and sera of psoriatic patients showed increased interferon activity [51,52]. Therefore, interferons were given to improve anti-infectious immunity. Interestingly, psoriasis plaques developed at the injection sites of recombinant IFN-γ [53]. Moreover, recombinant IFN-α treatment of patients with carcinomas or hepatitis led to exacerbation of concomitant psoriasis [54,55]. Recently, more detailed investigations showed increased expression of the Th1 cytokines IFN-γ, IL-2, tumor necrosis factor (TNF) and the type I chemokine IP-10 in psoriatic skin lesions, whereas

Th2 cytokines such as IL-4, IL-5 or IL-10 were absent or showed only limited expression [56-59]. $CD4^+$ T cells and $CD8^+$ T cells have been identified as the main source of IFN-γ in psoriatic lesions and this predominant Th1/Tc1 phenotype can also be detected in peripheral blood T cells of psoriasis patients [44,60]. Experimental work further support a role of $CD4^+$ Th1 cells in the pathogenesis of psoriasis. Anti-CD4 antibodies can suppress psoriasis, whereas reports on the use of anti-CD8 antibodies in the treatment of psoriasis are missing in the literature. [33,34]. Transfer of minor histocompatibility mismatched $CD4^+$ T cells but not of $CD8^+$ T cells into severe combined immunodeficiency (SCID) mice induced a psoriasiform skin disease [61]. Moreover, only CD4 positive but not $CD8^+$ T cell lines from peripheral blood of psoriasis patients induce psoriasis plaques in autologous skin engrafted onto SCID mice [62].

IMMUNE DEVIATION IN PSORIASIS

Immunosuppressive Cytokines

In agreement with the knowledge on the role of Th1 cells in the pathogenesis of psoriasis two approaches could be helpful to treat this inflammatory autoimmune disease without inducing general immunosuppression. The first approach is based on antagonists of Th1 effector cytokines to improve disease progression. Alternatively, the use of cytokines skewing disease-inducing Th1 cells into a Th2 phenotype may be helpful to treat psoriasis. TNF seem to be among the most important effector cytokines of Th1 cells in the pathogenesis of psoriasis. In consequence, general immunosuppression with neutralizing antibodies that block TNF are highly effective in the therapy of psoriasis [63,64]. Blocking TNF in the psoriatic network improves the disease effectively but increases the risk of infectious diseases [65]. The success of psoriasis therapies by blocking or neutralizing TNF underlines the pathological role of type I lymphocytes in psoriasis. A different cytokine, IL-10, inhibits activation and cytokine production of several immune cells, especially T cells. Therefore, IL-10 has been given to patients with psoriasis and this therapy may be useful in some patients. A placebo-controlled study could not show a significant efficacy of IL-10 in the therapy of psoriasis. However, IL-10 treatment resulted in a temporary clinical improvement in some patients [66]. More interestingly, the occurrence of Th2 responses has been observed in patients with subcutaneous application of IL-10, especially in those where psoriasis responded to the therapy [66-68].

Interleukin 4

Based on the investigations suggesting a central role of Th1 cells in the pathogenesis of psoriasis and based on the success obtained with therapeutic immune deviation in animal models of Th1-mediated autoimmune diseases, we initiated a trial on IL-4 therapy for psoriasis. IL-4 has been studied in more than 1000 cancer patients and did not cause major side effects at doses up to 3 μg/kg per day [69,70]. Psoriasis seems to be safe to test the concept of therapeutic immune deviation, as remissions can be obtained even in patients that have severe exacerbations. Moreover, in contrast to multiple sclerosis or rheumatoid arthritis, psoriasis seems to be mediated only by T cells, while Ig may be involved in the pathogenesis of the two other diseases. Therefore, a study was started to investigate the *in vivo* effects of IL-4 at doses that induce Th2 responses under *in vitro* conditions. Moreover, we analyzed the effect of systemic IL-4 application on $CCR5^+$ skin infiltrating Th1 cells. *In vitro* analysis revealed that human Th cells develop an IL-4-producing Th2 phenotype when stimulated with antigen in the presence of 100-1000 ng IL-4 per liter of culture medium [14,71]. To translate these conditions to an *in vivo* situation, we defined five groups of patients that were treated with 50 ng up to 500 ng IL-4 per kg body weight thrice daily. Eight patients with chronic psoriasis received low dose recombinant human (rh) IL-4 (50 – 100 ng/kg), a dose that is unlikely to induce Th2 cells under *in vitro* conditions. Twelve patients received high dose rhIL-4 (200 - 500 ng/kg) subcutaneously, a dose that should induce a Th2 phenotype in activated Th cells. The patients injected themselves rhIL-4 three times per day over a period of six weeks. Except of one patient in the high dose group, who developed fever, only minor side effects occurred during therapy. Importantly, no exacerbation of psoriasis was observed after the second week of rhIL-4 therapy. Instead, psoriasis improved in all patients in this first dose escalation study for psoriasis. After six weeks of therapy in 75% of the patients a more than 68% reduction of the Psoriasis Area and Severity Index (PASI) was observed and patients in the high dose rhIL-4 group improved significantly more than patients in the low dose group. Histology showed an overall significant decrease in epidermal thickness after six weeks of rhIL-4 treatment.

As a primary goal, we investigated the effects of rhIL-4 on the human immune system. Cytokine expression levels of IL-2, IL-4, IL-5, IL-8, IL-10, IL-12, IL-13, IL-19, IL-20, IL-20 receptor, TNF and IFN-γ have been analyzed before and after rhIL-4 therapy in skin by real time PCR and in peripheral blood. Significant changes have only been observed for the following parameters: In psoriatic skin a more than 10-fold decrease of the IFN-γ/IL-4 mRNA ratio has been detected in 3/8 patients in the high dose group. $CCR5^+$ Th1 cells of psoriasis lesions largely disappeared within the six weeks of IL-4 therapy from the dermis, whereas no change in $CCR5^+$ Th1 cells was observed within the epidermis. Furthermore, we found a strong reduction of IL-8 and IL19 mRNA in skin biopsies after six weeks of therapy. In the peripheral blood, we found a small but significant increase of 1 to 2% IL-4-producing $CD4^+$ T cells, in patients treated with high dose rhIL-4. Thus, clinical improvement of psoriasis was closely associated with the disappearance of Th1 cells in the skin and the appearance of $CCR5^-$ Th2-like cells, mainly in the dermis. In parallel, IL-4^+ $CD4^+$ T cells increased significantly in the blood. Even though one patient reported exacerbation of asthma at low dose rhIL-4 therapy, other Th2-related disorders, such as atopic reactions or increase of serum IgE levels did not occur in any of the patients. Together the data show that continuous systemic application of 0.2 – 0.5 μg / kg IL-4 thrice daily over a period of six weeks is capable of deviating a Th1 phenotype in the skin toward an anti-inflammatory Th2 phenotype, and possibly also in the peripheral blood. This first trial showing that immune deviation with rhIL-4 can improve an inflammatory autoimmune disease in humans suggests that immune

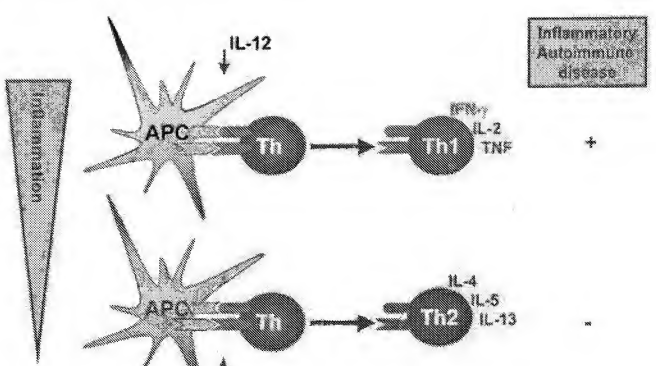

Fig. (1). Immune deviation of inflammatory Th1 cells into an anti-inflammatory Th2 phenotype by Interleukin 4, IL-11 or fumaric acid esters (FAE). Antigenpresenting cell (APC).

deviation may be promising for immunotherapies in human Th1-mediated autoimmune diseases [72,73]. Extended studies have to clarify the potency of rhIL-4 in the treatment of psoriasis and possibly other Th1-mediated autoimmune diseases.

Interleukin 11

A second cytokine that has the capacity to induce Th2 polarization in $CD4^+$ T cells is IL-11. Similar to IL-4, IL-11 can induce a Th2 phenotype *in vitro* and *in vivo* in $CD4^+$ T cells of human and mouse origin. IL-11 can induce Th2 polarization in human IL-11 receptor (IL-11R) positive CD4 positive T cells by inhibiting IFN-γ production and increasing IL-4 and IL-5 production [74]. The IL-11R complex is described on human and mouse $CD4^+$ and $CD8^+$ T lymphocytes. It is thought that IL-11 directly influences the differentiation of T lymphocytes [75]. In 1999 Trepicchio *et al.* performed a phase I dose escalation study on recombinant human (rh) IL-11, which was delivered subcutaneously to twelve patients with psoriasis. The patients were treated with 2.5μg/kg or 5.0μg/kg rhIL-11 every day for eight weeks. A 20 to 80% reduction of the PASI score was observed in eleven of the twelve patients during therapy. Seven of twelve patients showed a strong reduction of epidermal thickness, reduction of the number of lesional T cells and reduced ICAM-1 expression. Most importantly, patients responding to the rhIL-11 therapy, have a strong decrease of the IFN-γ/IL-4 ratio as measured by mRNA levels in lesional skin. In addition, two placebo-controlled trials have been performed with rhIL-11 for active Crohn's disease. The doses ranged from 5.0 to 40.0 μg/kg per week. The therapy was safe but clinical efficacy was only seen in a subset of patients [76,77]. No clinical benefit of rhIL-11 was seen in patients with rheumatoid arthritis [78]. Serious adverse events have not been reported in any of these studies using subcutaneous application of rhIL-11. However, the use of rhIL-11 in the therapy of human inflammatory autoimmune disease such as psoriasis or arthritis is limited due to the thrombocytopoietic effects of IL-11 [79]. It is likely that this potential complication may halt from further investigation of rhIL-11 as an anti-inflammatory drug.

Fumaric Acid Esters

Unexpectedly, fumaric acid esters (FAE), a novel anti-psoriatic drug seem to affect the immune system similar to IL-4 or IL-11. FAE have been used for about 15 years in the therapy of psoriasis in some European countries [80]. Several double-blind, placebo controlled studies have proven safety, also for long-term therapy, and efficacy of FAE in the therapy of psoriasis [81]. Side effects such as flushing and diarrhea are common at higher doses of FAE and in some patients leukopenia and eosinophilia may occur. In humans FAE seem to have the potential to induce Th2 cytokines such as IL-4 and IL-5, and suppress type Th1 cytokines,

such as IFN-γ *in vitro* and *in vivo* [82,83]. FAE seem to count among the most effective therapies of psoriasis [27].

Even though FAE are used for more than fifteen years, the mechanisms underlying the improvement of psoriasis remain poorly understood. Today it is believed that only one of the components of FAE, dimethylfumarate and its metabolite methylhydrogen fumarate, induces the immunological changes observed during FAE therapy. FAE can reduce the number of peripheral blood lymphocytes and induce apoptosis in dendritic cells. Nonetheless, psoriasis improves in most of the patients without causing important leukopenia or lymphopenia. It is still open whether FAE really reduce T cells causing psoriasis, and whether FAE alter homing of T lymphocytes into tissues such as spleen, lymph nodes or skin. Cytokine analysis strongly suggests that in addition to these two effects, FAE can promote Th2 cells producing IL-4, IL-5 and IL-10, but little IFN-γ.

CONCLUSIONS

The data reported strongly suggest that deletion of autoreactive T cells is effective in the treatment of psoriasis. This benefit has to be balanced against the risks of tumors or infections. In sharp contrast, immune deviation seems to be highly antigen-specific, and therefore should avoid the side effects mentioned above. Even though IL-4-induced immune deviation is highly promising the mode of action is still unclear. Most likely, deviation of disease-inducing T cells by IL-4 from a Th1 into a Th2 phenotype improves the disease. In psoriasis this will remain an open question until the involved autoantigens are not identified. If efficiency is confirmed by larger studies, immune deviation of autoreactive Th1 cells into Th2 cells may be used to develop vaccination strategies against psoriasis and possibly also against other inflammatory autoimmune diseases.

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EXHIBIT B

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$CD3^+CD56^+$ NK T cells are significantly decreased in the peripheral blood of patients with psoriasis

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SUMMARY

Psoriasis is a chronic, inflammatory, hyperproliferative skin disease, in which autoimmunity plays a great role. Natural killer T cells (NK T cells), are suggested to be involved in the pathogenesis of different autoimmune diseases. To examine the involvement of $CD3^+CD56^+$ NK T cells in the pathogenesis of psoriasis, we investigated the lymphocyte subpopulations obtained from blood samples of psoriatic patients before and after treatment, and of healthy controls, using two-colour flow cytometry. We found no significant differences between total T cells, total B cells, T helper cells, T cytotoxic cells and NK cells in patients with psoriasis before and after treatment and in controls. Increased percentage of memory T cells and decreased percentage of naive T cells was detected in psoriatic patients compared to controls, but these changes were not statistically significant. The $CD3^+CD56^+$ cells of psoriatic patients were significantly decreased relative to controls. The percentage of $CD3^+CD56^+$ cells increased after different antipsoriatic therapies, but remained significantly lower than those found in controls. $CD3^+CD56^+$ cells of healthy controls were capable of rapid activation, while in psoriatic patients activated NK T cells were almost absent. The decrease in the number of $CD3^+CD56^+$ cells may represent an intrinsic characteristic feature of patients with psoriasis, which is supported by the fact that after treatment NK T cells do not reach the values found in controls. In conclusion our results suggest that $CD3^+CD56^+$ NK T cells could be actively involved in the development of Th1 mediated autoimmune diseases.

Keywords $CD3^+CD56^+$ NK T cells autoimmunity psoriasis

INTRODUCTION

Although the pathogenesis of psoriasis is not yet clear, there are characteristic features of the disease which suggest an immunological mediated process. Several direct and indirect evidences suggest that T cells play a crucial role in the pathogenesis of psoriasis [1–7]. The presence of T helper cells that secrete type 1 cytokines (IFN-γ, IL-2, TNF-α), was demonstrated in psoriatic skin lesions [8–13]. A type 1 differentiation bias was also observed in circulating blood T cells of psoriatic patients [14]. The existence of an imbalance between Th1 and Th2 cells in psoriasis was supported further by findings which demonstrated that IL-10 was decreased in psoriatic lesions [15]. Moreover, during antipsoriatic therapy an increase in IL-10 mRNA expression was observed in peripheral blood mononuclear cells [16]. IL-10 therapy given either intralesionally or subcutaneously resulted in marked reduction of psoriatic lesions [16,17]. These data suggest that psoriasis is an inflammatory Th1 mediated autoimmune disorder, but the triggering autoantigens are still not identified.

Although the factors that induce the imbalance between Th1 and Th2 cells in psoriasis are unknown, a possible role could be attributed to natural killer T cells (NK T cells) [18]. NK T cells are a heterogeneous T cell population characterized by the co-expression of αβ or γδ TCRs and various NK receptors, including CD16, CD56, CD161, CD94, CD158a and CD158b [19–21]. NK T cells have the ability to rapidly secrete large amounts of cytokines following activation [22–24]. NK T cell clones secrete type 1, type 2 or both types of cytokines, which could influence the differentiation of Th0 cells towards Th1 or Th2 cells [25,26]. $CD3^+CD56^+$ cells represent one of the NK T cell populations.

The number of $CD3^+CD56^+$ NK T cells has been shown to be significantly decreased in the peripheral blood of patients with

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rheumatoid arthritis, another Th1 mediated autoimmune disease [27].

In the present study we determined the number of $CD3^+CD56^+$ NK T cells in the peripheral blood of patients with psoriasis (before and after treatment) and in healthy controls. Other lymphocyte subpopulations (total T cells, T helper cells, T cytotoxic cells, T memory cells, T naive cells, B lymphocytes and NK cells) were also analysed and compared in psoriatic patients and healthy volunteers.

Our results show that $CD3^+CD56^+$ NK T cells are significantly decreased in the peripheral blood of patients with psoriasis relative to the healthy controls. This finding is discussed in relation with the development of a type 1 immune response in psoriasis.

PATIENTS AND METHODS

Patients' profile

Peripheral blood samples of 15 patients with psoriasis (one erythrodermic, two guttate type and 12 chronic plaque psoriasis) were obtained with informed consent for use in this study. The Ethical Committee of the University of Szeged, Hungary approved this investigation. Blood samples were collected before and after treatment from all patients. The patients' characteristics are presented in Table1. Therapeutic modalities included monotherapy and combined therapeutic regimens (Table2). The second blood sample was taken at the time when the applied treatment regimen had been completed. At this time the majority of patients was symptom-free or had minimal skin changes (PASI < 4).

The control group consisted of 12 healthy hospital employees (seven females and five males), aged 37·08 ± 7·21 years with informed consent for use in the study. In the control group nobody took any medication and nobody suffered from any known acute or chronic disease.

Reagents

Anti-CD3 FITC, anti-CD3 PE (clone UCHT1), anti-CD19 PE (clone HD37), anti-CD4 FITC (clone MT310), anti-CD8 PE, anti-CD8 FITC (clone DK 25), anti-CD25 FITC (clone ACT-1) and isotype-matched labelled mouse immunoglobulins were obtained from DAKO (Copenhagen, Denmark), anti-CD56 FITC (clone NKH-1), anti-CD45RA PE (clone F8-11–13) were obtained from Serotec (Oxford, UK), anti-CD4 FITC (clone SK3), anti-CD56 PE (MY31) from Becton-Dickinson (San Jose, CA, USA) and anti-CD45RO FITC (clone UCHL1) was obtained from Immunotech (Beckman Coulter, Fullerton, CA, USA).

Table 1. Patient profile

Sex (male : female)	12 : 3*	
Age (years)	42·35 ± 14·32	(20–67)‡
Age of onset (years)	25·7 ± 10·38†	(9–45)
Disease duration (years)	16·7 ± 9·21	(3–37)
Psoriasis type (chronic : eruptive)	13 : 2	
PASI score	18·77 ± 12·25	(6–54)
Family history (positive : negative)	6 : 9	

*Number of patients; †mean ± s.d.; ‡range.

Immunostaining and flow cytometry

Peripheral blood, anticoagulated with EDTA, was collected. Each blood sample (50 μl) was stained with two monoclonal antibodies, one conjugated with FITC and the other with PE (10 μl from each) at room temperature, in the dark for 20 min. Erythrocytes were lysed with FACS Lysing Solution (Becton Dickinson San Jose, CA, USA). After two washes with PBS the cells were resuspended in PBS for immediate analyses or were fixed with 2% paraformaldehyde for overnight storage before analyses. Two-colour flow cytometry was performed by using a FACSCalibur cytometer and the data were analysed using Cell Quest software (Becton Dickinson, San Jose, CA, USA). In each stain 30 000 events were acquired.

Isolation and stimulation of T cells from peripheral blood

Mononuclear cells (PBMC) were isolated from peripheral venous blood samples of psoriatic patients and healthy controls by Ficoll-Hypaque density gradient centrifugation (Biotech Inc, Piscataway, NJ, USA). PBMC were recovered at the interface and washed in PBS supplemented with 2% fetal calf serum (FCS). T cells were isolated by positive selection using uniform magnetizable polystyrene beads coated with monoclonal antibodies specific for CD3 (Dynabeads M-450 CD3 from Dynal, Oslo, Norway) and a magnetic particle separator (Dynal MPC, from Dynal, Oslo, Norway) following the protocol provided by the manufacturer. Briefly, PBMC were incubated with CD3 monoclonal antibody coated magnetic beads (bead : cell ratio 5 : 1, Dynabeads concentration 1×10^7/ml) for 6 h in RPMI 1640 culture medium supplemented with 10% FCS, at 37°C in a humid 5% CO_2 incubator. After the incubation time Dynabeads were detached from the cells by pipetting the cell suspension 10 times through an automated pipette. The beads were than removed from the cell suspension using a Dynal MPC. The beads attached to the tube wall while the cells remained in the suspension. This isolation procedure through the CD3 binding results in stimulation of the $CD3^+$ cells [28]. The isolated and stimulated cells were then analysed by two-colour flow cytometry, using monoclonal antibodies for

Table 2. Treatment regimens used in the study

Treatment	Number of patients
Dithranol†	6
PUVA*	6
Dithranol + PUVA‡	1
Dithranol + narrowband ultraviolet B (311 nm)§	1
Re-PUVA¶	1

†Dithranol treatment was used in slowly increasing concentrations starting with 0·5% up to 8% depending on the induced erythema. *Oral 8-methoxypsoralen + UVA (four times a week) was administered from 1 J/cm^2 up 5 J/cm^2 depending on the induced erythema. ‡Dithranol treatment with slowly increasing concentrations starting with 0·5% up to 8% was combined with oral 8-methoxypsoralen + UVA (four times a week) from 1 J/cm^2 up 5 J/cm^2 depending on the induced erythema. §Dithranol treatment with slowly increasing concentrations starting with 0·5% up to 8% was combined with narrowband ultraviolet B from 0·2 J/cm^2 up to 2·2 J/cm^2 (five times a week) depending on the induced erythema. ¶Oral acitretin (0·5 mg/kg body wt) + oral 8-methoxypsoralen + UVA (four times a week) from 1 J/cm^2 up 5 J/cm^2 was administered depending on the induced erythema.

detection of CD56, CD25, CD4 and CD8. After 6 h of anti-CD3 antibody stimulation, the CD3 antigen was transiently down-regulated. By growing the cells for further 24 h in culture medium the CD2 antigen was reexpressed on the cell surface, and could be detected using FITC conjugated CD3 antibodies. Vitality of isolated cells was tested by trypan blue exclusion.

Statistical analysis

Statistical analysis of the data was made by ANOVA, Pearson correlation test and Spearman's rank order correlation test. For significant ANOVA values, groups were compared by Tukey's *post hoc* test for multiple comparisons with unequal cell size. A probability level of 0·05 was accepted as indicating significant differences.

RESULTS

Analyses of lymphocyte subsets

The lymphocyte subsets in patients with psoriasis before treatment and in healthy controls were analysed. The percentage of $CD3^-CD56^+$ NK T cells was significantly decreased in the peripheral blood of patients with psoriasis before treatment compared with healthy controls (1·79 ± 1·07% in patients *versus* 5·22 ± 1·74% in controls, $P < 0·0001$) (Table 3). Representative flow cytometric analyses show $CD3^-CD56^+$ NK T cells in a patient before treatment (0,78%) (Fig. 1b) *versus* in a healthy control (6·28%) (Fig. 1a). The absolute number of circulating $CD3^-CD56^+$ NK T cells was also significantly lower in psoriatic patients before treatment than the values found in healthy controls (29·43 ± 17·12 μl in psoriasis before treatment *versus* 120·15 ± 45·35 μl in controls, $P < 0·0001$). Memory T cells ($CD3^+CD45RO^+$) represented a larger (35·36 ± 9·38% in patients *versus* 27·21 ± 7·34% in controls) and naive T cells ($CD3^+CD45RA^+$) a smaller population (37·71 ± 8·34% in patients *versus* 45·00 ± 7·19%) in the peripheral blood of patients relative to controls; however, these differences did not reach statistical significance. Similarly, a slight but statistically not significant increase in the proportion of helper CD4+ T helper cells was observed in patients with psoriasis (44·52 ± 9·05% in patients *versus* 38·97 ± 5·66% in controls $P > 0·05$). There was no difference in the percentages of B lymphocytes, conventional NK cells ($CD3^-CD56^+$), total T cells and T cytotoxic $CD8^+$ cells between the two groups (Table 3).

Table 3. Percentage of lymphocyte subsets (mean ± s.d.) in peripheral blood of patients with psoriasis before treatment, after treatment and of healthy controls

	Psoriasis before treatment (%)	Psoriasis after treatment (%)	Healthy controls (%)
$CD3^+$	72·46 ± 8·59	70·04 ± 8·59	70·78 ± 4·71
$CD19^+$	11·24 ± 4·87	11·5 ± 5·41	13·97 ± 4·63
$CD3^+CD4^+$	44·52 ± 9·05	42·54 ± 8·22	38·97 ± 5·66
$CD3^+CD8^+$	27·12 ± 8·21	26·39 ± 7·18	28·95 ± 7·43
$CD3^+CD56^+$	1·79 ± 1·07	2·68 ± 1·04	5·22 ± 1·74
$CD3^-CD56^+$	10·20 ± 5·69	12·47 ± 6·98	10·30 ± 4·7
$CD3^+CD45RA^+$	37·71 ± 8·34	37·90 ± 8·60	45·00 ± 7·19
$CD3+CD45RO^+$	35·36 ± 9·38	35·13 ± 9·87	27·21 ± 7·34

Comparing the lymphocyte subsets of patients with psoriasis before and after treatment, the only lymphocyte population in which changes were statistically significant was the NK T ($CD3^+CD56^+$) subset. Both the percentage and the absolute cell number of NK T cells were significantly increased in the peripheral blood of patients with psoriasis after treatment (2·68 ± 1·04%, 29·43 ± 17·12 μl *versus* 1·79 ± 1·07%, 58·95 ± 31·56 μl, $P < 0·001$), but did not reach the values found in healthy controls (Table 3). A representative flow cytometric analysis shows the comparison of $CD3^+CD56^+$ NK T cells in a patient before treatment (0·78%) (Fig. 1b) *versus* in a patient after treatment (3·04%) (Fig. 1c). We found no statistically significant changes in the other lymphocyte subsets (Table 3). The antipsoriatic treatments used in this study had no effect on the number of memory and naive T cells (Table 3).

After treatment the absolute cell number of $CD3^+CD56^+$ NK T cells remained significantly decreased in patients with psoriasis compared to healthy controls (58·95 ± 31·56 μl in patients after treatment *versus* 120·15 ± 45·35 μl in controls, $P < 0·001$). The same was observed when the percentage of NK T cells among lymphocytes was analysed in peripheral blood samples (2·68 ± 1·04% in patients *versus* 5·22 ± 1·74% in controls, $P < 0·001$). (Table 3). Representative flow cytometric analyses show $CD3^+CD56^+$ NK T cells in a patient after treatment (3·04%) (Fig. 1c) *versus* in a healthy control (6·28%) (Fig. 1a). Memory T cells remained elevated (35·13 ± 9·87% in patients *versus* 27·21 ± 7·34% in controls) and naive T cells were decreased in the psoriasis group compared to healthy controls (37·90 ± 8·60% in patients *versus* 45·00 ± 7·19% in controls) (Table 3), without reaching statistical significance. Similarly, the number of T helper cells showed a slight, statistically not significant elevation in treated patients compared to controls (42·54 ± 8·22% in patients *versus* 38·97 ± 5·66% in controls $P > 0·05$) (Table 3). No difference between the two groups was found regarding B lymphocytes, NK cells, total T cells and cytotoxic T cells (Table 3).

$CD3^+CD56^+$ NK T cells and patients' profile

To determine whether the number or the percentage of $CD3^+CD56^+$ NK T cells in patients with psoriasis shows any correlation with age, PASI score and disease duration the appropiate correlation test has been applied. The analysis of the possible correlation of NKT cells with age was also performed in healthy controls. Statistical analyses of our data showed a slight, but significant direct correlation between NK T cells and the age of controls ($r_s = +0·39$). In contrast in psoriatic patients a slight, but statistically significant inverse correlation ($r_s = -0·31$) was detected (data not shown). We observed that patients with a long-term history of frequent relapses, who did not respond well to treatment, had the lowest NKT cell counts. In these patients the recovery of NK T cells following therapy was slower and generally poor. However, $CD3^+CD56^+$ cells showed no correlation with PASI score and disease duration (data not shown).

Stimulation of peripheral blood T cells with anti-CD3 monoclonal antibodies

To examine the activation status of $CD3^+CD56^+$ NK T cells, we separated T cells from the peripheral blood of healthy controls and of patients with psoriasis. These T cells were stimulated for 6h using anti-CD3 monoclonal antibodies, and then flow cytometric analysis was performed. On the FSC/SSC dot-plot of separated and stimulated T cells obtained from healthy controls a

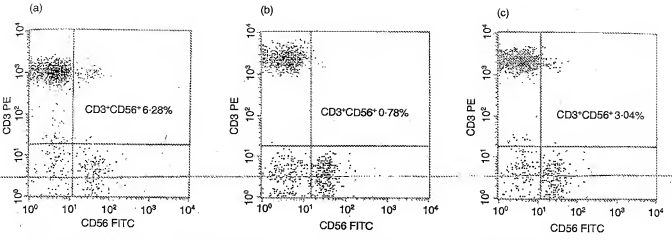

Fig. 1. Representative dot-plot diagrams of peripheral blood cells stained with PE conjugated anti-CD3 monoclonal antibodies and FE conjugated anti-CD56 monoclonal antibodies. $CD3^+CD56^+$ NK T cells in a healthy control (a), in a patient with psoriasis before treatment (b) and in the same patient after treatment (c) are in the upper-right quadrants of each dot-plot diagrams. $CD3^+CD56^+$ NK T cells are significantly decreased in the peripheral blood of patients with psoriasis (b, 0·78%), the percentage of these cells increases after therapy (c, 3·04%), but remains significantly lower than in healthy controls (a, 6·28%).

distinct cell population was recognized, which was not present as a distinct population on the dot-plot of unstimulated $CD3^+$ T cells (Fig. 2). These cells showed a marked granulated pattern. Analysis of stimulated T cells was performed by using two gates: R1 for these granulated cells and R2 for the other cells. All the granulated cells (gate R1) expressed the surface molecule CD56, but only a minority of the other cells (gate R2) expressed this molecule (Fig. 3). Thus the granulated cells were NK T ($CD3^+CD56^+$) cells. More than half of NK T cells (gate R1) expressed the CD4 marker (71·25%), while very few of the less granulated $CD3^+CD56^+$ NK T cells (gate R2) were $CD4^+$ (0·58%) (Fig. 3). The CD8 molecule was expressed by more than half of the less granulated $CD3^+CD56^+$ NK T cells (gate R2) and by about one-third of the granulated NK T cells (gate R1) (Fig. 4). The low affinity receptor for IL-2, an early activation marker for T cells, was detected with an anti-CD25 FITC labelled monoclonal antibody. Almost all the granulated NK T cells (gate R1) expressed CD25 molecules (93·2%), indicating that they were activated cells. Among the less granulated cell population (gate R2) only 59·65% of the cells expressed the low affinity IL-2 receptor (data not shown). After analysing the separated and stimulated T cells collected from patients with psoriasis we found that $CD56^+$ T cells with marked granulated pattern were almost absent. Scattered $CD56^+$ T cells were present between the cells with normal granulation pattern, characteristic for lymphocytes (data not shown). These findings are in concordance with the low levels of $CD3^+CD56^+$ NK T cells that were detected in the peripheral blood samples of patients with psoriasis by flow cytometric analyses of unseparated cells.

In each experiment controls staining of separated and stimulated T cells was performed after 24 h of culture in medium alone, using FITC conjugated anti-CD3 antibodies. The percentage of separated cells that expressed the surface molecule CD3 was 95–98% (data not shown). The vitality of separated and stimulated cells was tested using trypan blue, and was always above 95%.

DISCUSSION

NK T cells are phenotypically and functionally diverse [28]. Initially, NK T cells were described as cells that express an invariant TCR Valpha14 in mouse and Valpha24 in humans [29]. Recently NK T cells expressing diverse TCRs have been also recognized [22,23,30]. The $CD3^+CD56^+$ cells represent one of these NK T cell subpopulations.

Our results show that the number of $CD3^+CD56^+$ NK T cells is significantly decreased in the peripheral blood of patients with psoriasis and that the percentage of these cells increases after different therapies used in psoriasis, but remains significantly lower than those found in healthy controls. The full relevance of this finding is still speculative. The decrease in the number of $CD3^+CD56^+$ cells may represent an intrinsic characteristic feature of patients with psoriasis. This hypothesis is supported by the fact that NK T cells do not reach the values found in healthy controls, so it is possible that the percentage of this cell population is permanently decreased in the peripheral blood of patients with psoriasis. Another possible cause that leads to decreased number of NK T cells may be represented by the early activation of these cells by antigens involved in the relapse of the disease, followed by apoptosis [31]. Since we did not study NK T cells in the skin we could not exclude that the decrease in NK T cells in the peripheral blood might come from the differential homing of NK T cells to the skin lesions. Further studies are needed to elucidate the direct role of NK T cells in the skin of patients with psoriasis.

In our study we found that $CD3^+CD56^+$ NK T cells were capable of rapid activation. After stimulation of $CD3^+$ T cells one population of activated NK T cells appeared. NK T cells are able to recognize non-peptide antigens [32]. *In vivo* administration of synthetic ceramide induces the secretion of both type 1 and type 2 cytokines [24], but repeated doses polarize NK T cells towards Th2 cytokine synthesis [33,34]. Thus IL-4 produced by the NK T cells plays a major role in promoting the differentiation of Th0 cells into Th2 cells [34,35]. Although the precise role of NK T cells

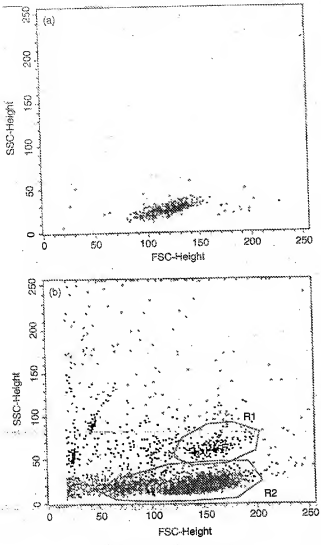

Fig. 2. Light scatter analysis (FSC *versus* SSC) of unstimulated peripherial blood $CD3^+$ T cells (a) and of separated and stimulated $CD3^+$ T cells (b). Peripheral venous blood sample was obtained from a healthy control. Cell separation and stimulation was performed using magnetic beads coated with anti-CD3 monoclonal antibodies. After stimulation of the $CD3^+$ T cells a distinct population of granulated cells appeared (R1).

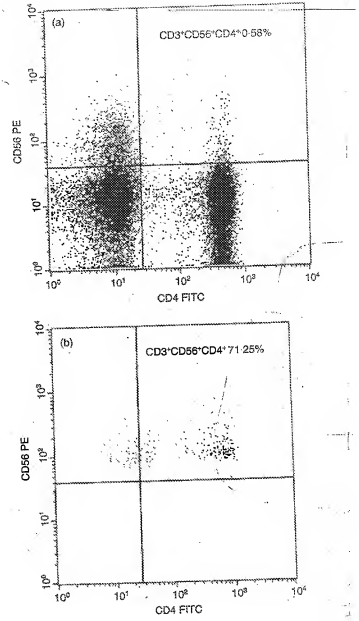

Fig. 3. Phenotypic analyses of separated and stimulated $CD3^+$ T cells. T cells were stained with PE conjugated anti-CD56 and FITC conjugated anti-CD4. The expression of CD56 and CD4 surface molecules on granulated $CD3^+$ T cells, gate R1 in Fig. 2b and on the less granulated $CD3^+$ T cells, gate R2 in Fig. 2a are shown. All the granulated $CD3^+$ T cells express the surface molecule CD56 (b, upper left and right quadrants), but of the less granulated $CD3^+$ T cells only a minor population express this molecule (a, upper left and right quadrants). 71·25% of granulated $CD3^+CD56^+$ cells express the CD4 marker (b, upper right quadrant), at the same time very few (0·58%) of the less granulated T cells express both CD56 and CD4 on the surface (a, upper right quadrant).

is not yet elucidated, they can be a regulatory cell type playing a pivotal role in the development of peripheral tolerance and in the modulation of immune responses by inducing a shift in early activation and consequently in the cytokine secretion of classical T cells [36,37]. Psoriasis is an autoimmune disease in which type 1 cytokine secretion pattern can be demonstrated in T cells derived from lesional skin and from peripheral blood [8,14]. It is possible that this feature is a consequence of an inefficient type 2 response, because of lack of NK T cells. This issue might be clarified by comparing the IL-4 producing capacity of $CD3^+CD56^+$ NK T cells from psoriatic patients with that from healty controls. However, the study of the cytokine production in psoriasis on a per cell basis was beyond the scope of our present work. Our results are the first clear-cut evidence showing that low NK T cell counts are a characteristic of psoriasis patients.These findings raised the question of whether NK T cell deficiency might result in the missing contraregulatory signals needed for the development of a normal immune response upon antigen stimulation, and favours excessive activation of Th1 cells. Type 2 cytokines are also important in the development of tolerance [38]; thus deficit in these cytokines can favour the development of autoimmunity.

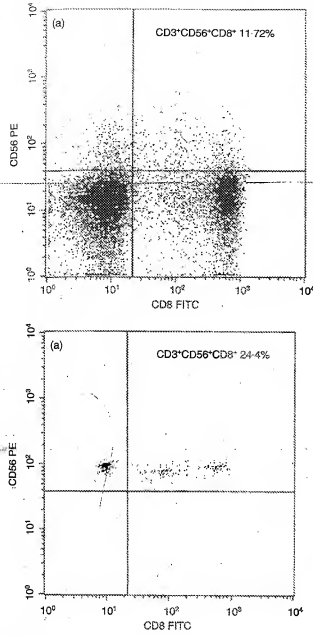

Fig. 4. Phenotypic analyses of separated and stimulated $CD3^+$ T cells. T cells were stained with PE conjugated anti-CD56 and FITC conjugated anti-CD8. The expression of CD56 and CD4 surface molecules on granulated $CD3^+$ T cells, gate R1 in Fig. 2b and on the less granulated $CD3^+$ T cells, gate R2 in Fig. 2a are shown. 24·4% of granulated $CD3^+CD56^+$ cells express the CD8 marker (b, upper right quadrant), at the same time 11·72% of the less granulated T cells express both CD56 and CD8 on the surface (a, upper right quadrant).

The dysfunction of NK T cells correlates with the pathogenesis of other T cell-mediated autoimmune diseases [39,40]. In lpr/lpr mice, in which a spontaneous autoimmune syndrome resembling human systemic lupus erythematosus occurs, NK T cells disappear from the periphery by the time the autoimmune disease develops. Selective experimental depletion of NK T cells from the peripheral blood results in early onset and exacerbation of the autoimmune phenomena [39]. The selective reduction of NK T cells has been also detected in non-obese diabetic mice [40,41]. Studies in humans have showed that decreased number of NK T cells are present in the peripheral blood of patients with rheumatoid arthritis, systemic sclerosis and insulin-dependent diabetes mellitus [27,42,43].

Other lymphocyte populations were also investigated. We found no significant differences between total T cells, total B cells, T helper cells, T cytotoxic cells and NK cells in patients with psoriasis and healthy controls. These results are in concordance with observations of other authors and highlight further the significance of $CD3^+CD56^+$ NK T cells in the pathogenesis of psoriasis [44–46].

We found increased percentage of memory T cells and decreased percentage of naive T cells. However, these changes were not statistically significant, they might be related to the chronic activation of the immune system in patients with psoriasis. Increased numbers of memory T cells have been found in lesional skin and in the synovial tissue of patients with psoriatic arthritis [38,47]. It is interesting that the treatments used in this study had no effect on memory and naive T cells. It is possible that the persistence of the increased number of memory T cells is one of the factors that contribute to the relapses observed in psoriasis, but these assumptions are still speculative and need further investigations.

In conclusion, our results suggest that $CD3^+CD56^+$ NK T cells have a role in the pathogenesis of psoriasis and that reduced NK T cells can be of importance in the development of psoriasis, a Th1 mediated autoimmune disease.

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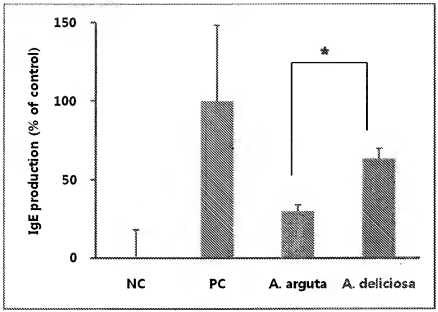

* $p<0.05$

NC = Negative Control

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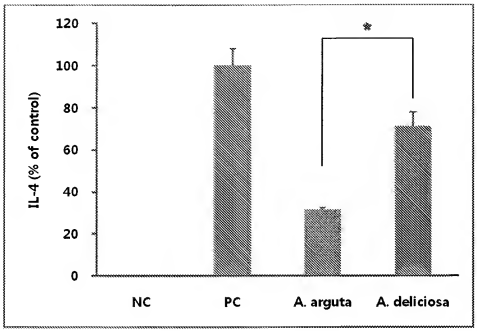

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